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Inhibitors of nicotinamide *N*-methyltransferase designed to mimic the methylation reaction transition state

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Nicotinamide *N*-methyltransferase (NNMT) is an enzyme that catalyses the methylation of nicotinamide to form *N*'-methylnicotinamide. Both NNMT and its methylated product have recently been linked to a variety of diseases, suggesting a role for the enzyme as a therapeutic target beyond its previously ascribed metabolic function in detoxification. We here describe the systematic development of NNMT inhibitors derived from the structures of the substrates involved in the methylation reaction. By covalently linking fragments of the NNMT substrates a diverse library of bisubstrate-like compounds was prepared. The ability of these compounds to inhibit NNMT was evaluated providing valuable insights into the structural tolerances of the enzyme active site. These studies led to the identification of new NNMT inhibitors that mimic the transition state of the methylation reaction and inhibit the enzyme with activity on par with established methyltransferase inhibitors.

Introduction

Nicotinamide *N*-methyltransferase (NNMT) is an enzyme that transfers a methyl group from *S*-adenosyl-L-methionine (AdoMet) to nicotinamide (NA) forming *N*'-methylnicotinamide (MNA) and *S*-adenosyl-L-homocysteine (AdoHcy) (Fig. 1). The enzyme has gained much interest in the last decade because of its involvement in a variety of diseases. Whereas 30 years ago, the enzyme was thought to function exclusively in detoxification and metabolic pathways, it has now become clear that the enzyme has far more complex roles in both healthy and disease states. In many types of cancer, NNMT is overexpressed and it has the potential as a biomarker for gastric, oral, renal and lung cancers.^{1–4} In this regard, the overexpression of NNMT has recently been shown to cause AdoMet depletion in cancer cells.⁵ Other studies have suggested a role for NNMT as a cytoprotective agent in Parkinson's disease^{6–8} while other findings point to a potential link between MNA and lifespan regulation.⁹ In addition, the upregulation of NNMT is observed in a range of metabolic disorders including diabetes and obesity.^{10–12} Despite its implication in many disease states, very few inhibitors of NNMT have been described to date.^{13,14} Therefore, we set out to develop inhibitors of NNMT based on a bi-substrate

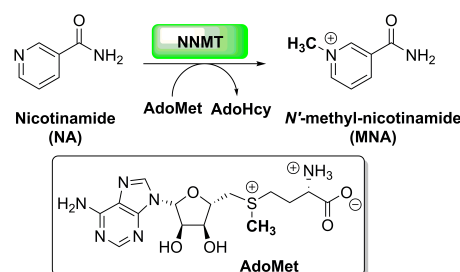


Figure 1. Methylation of nicotinamide (NA) by NNMT using *S*-adenosyl-L-methionine (AdoMet) as the methyl donor, forming *N*'-methylnicotinamide (MNA) and *S*-adenosyl-L-homocysteine (AdoHcy)

transition state mimic approach. The recently reported NNMT crystal structure reveals the interactions of the substrates with the active site residues (Fig. 2).¹⁵ The active site can be roughly divided into three binding pockets: one for the adenosine group (A), one for the amino acid moiety (B) and one for nicotinamide unit (C). In designing the inhibitors here described we focused on the structural elements of the substrates implicated in the transition state of the methylation reaction.

By linking fragments of the NNMT substrates known to interact with the different active site pockets, bisubstrate-like inhibitors were obtained and their inhibitory activity evaluated. The first series of inhibitors examined were simple *N*-alkylated nicotinamide analogues. In a second series, we coupled the adenosine moiety to the nicotinamide ring via a range of spacers. In a third approach, we omitted the adenosine group and instead coupled nicotinamide to an amino acid moiety again with a variety of spacers. Finally, we also synthesized a "trivalent" compound comprising the

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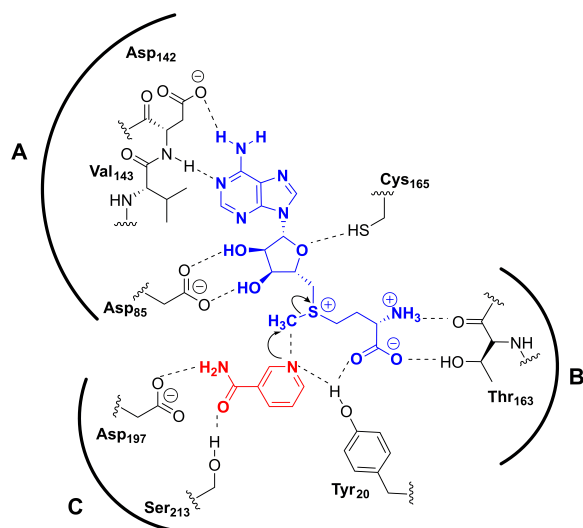


Figure 2. Schematic overview of the methylation reaction transition state of NNMT showing the structures of substrates AdoMet in blue and nicotinamide in red and their interactions with the active site residues. The active site can be divided into three binding pockets: adenosine pocket A; amino acid pocket B and nicotinamide pocket C.

nicotinamide, adenosine and amino acid moieties with the aim of simultaneously interacting with all three binding pockets. To assess NNMT inhibition, we used an assay recently developed in our group.¹⁶ This assay employs Ultra High Performance (UHP) Hydrophilic Liquid Interaction Chromatography (HILIC) Quadrupole Time-Of-Flight Mass Spectrometry (QTOF-MS) to rapidly and efficiently measure NNMT activity. Using this method, we previously determined the IC_{50} values for the general methyltransferase inhibitors sinefungin and AdoHcy and here demonstrate that it can be used for the evaluation of new NNMT inhibitors. In the sections below we describe the synthesis of the different NNMT inhibitors designed and discuss their inhibitory activity.

Results and Discussion

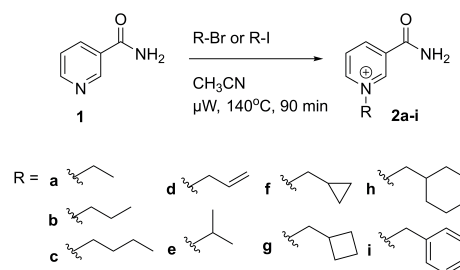
The inhibitors described in the present study all contain a nicotinamide mimic and were designed to approximate the NNMT transition state. The different inhibitors here explored each build upon the nicotinamide mimic which was elaborated to include structural elements of the adenosine and/or amino acid moieties of the AdoMet cofactor. The NNMT inhibition of all compounds prepared was first assessed at a fixed concentration of 250 μ M. In cases where at least 50% inhibition was detected at this concentration full inhibition curves were measured in triplicate to determine the corresponding IC_{50} values.

The first set of compounds studied were prepared directly from nicotinamide, which was *N*-alkylated with ethyl, propyl, isopropyl, allyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and benzyl-groups (Scheme 1). For the synthesis of compounds **2a-i**, we developed a convenient microwave-assisted alkylation procedure followed by straightforward purification of the alkylated products via precipitation. When testing the positively charged *N*-alkylated nicotinamides as

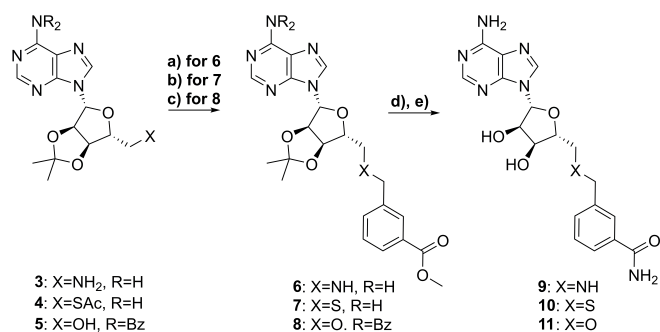
NNMT inhibitors, we found that *N*-alkylation of nicotinamide with any group bigger than a methyl group – as present in MNA – lead to inactive compounds. While MNA was found to have an IC_{50} value of 24.6 ± 3.2 μ M (Table 1), none of the other alkylated compounds exhibited NNMT inhibition at 250 μ M.

The next series of NNMT inhibitors pursued were based on a bisubstrate strategy. This approach is similar to that recently described by our group in preparing inhibitors of protein arginine *N*-methyltransferases¹⁷. To this end, an adenosine moiety was coupled to a nicotinamide mimicking group via a series of different linkers (Scheme 2). Unlike the positively charged *N*-alkylated compounds prepared in the first series (Scheme 1), the bisubstrates were prepared as neutral compounds wherein the pyridine of nicotinamide is replaced with a benzene core. In examining the NNMT transition state model (Fig. 2), it appears that the spacing between the ribose group of the adenosine moiety and the nitrogen of nicotinamide is 3 atoms. We therefore synthesized three-atom spaced conjugates of adenosine and benzamide via *N*-, *O*- and *S*-linkages resulting in compounds **9-11**.

Intermediate compound **6** was prepared via reductive amination of free amine **3** with methyl 3-formylbenzoate, followed by microwave-assisted amidation of the methyl ester using concentrated ammonium hydroxide in methanol. A final deprotection step using TFA and water and subsequent purification by preparative HPLC gave compound **9**. For the preparation of thioether bisubstrate analogue **10**, intermediate compound **7** was first prepared via a one-pot deprotection of *S*-acetylated thiol **4** followed by direct alkylation with methyl-3-bromomethylbenzoate. Subsequent amidation, deprotection, and purification yielded compound **10**. For the synthesis of oxy-ether linked conjugate **11** the exocyclic adenine amine was dibenzoylated to avoid overalkylation by methyl-3-bromomethylbenzoate under the strongly basic conditions necessary for ether formation. Following *O*-alkylation, intermediate **8** was amidated followed by deprotection and purification to yield **11**. Somewhat surprisingly, bisubstrate analogues **9-11** showed very little NNMT inhibition at a concentration of 250 μ M. This lack of inhibition may be explained by both the omission of the pyridine nitrogen as well as the absence of interactions with the amino acid binding pocket. This explanation is supported by the significantly reduced enzymatic activity previously reported for the Y20A mutant of NNMT.¹⁵ As seen, in the



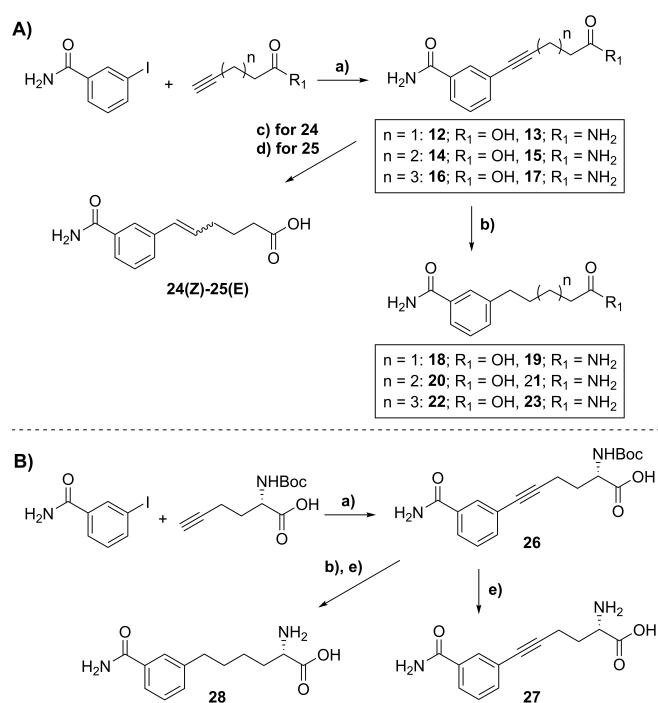
Scheme 1. Synthesis of alkylated nicotinamides **2a-i** via microwave-assisted coupling of alkyl bromides or iodides. *Reagents and conditions:* a) alkyl iodide or alkyl bromide, CH_3CN , μW , $140^\circ C$, 1.5h, 29-92%.



Scheme 2. Synthesis of the adenosine-nicotinamide conjugates **9–11**. *Reagents and conditions:* a) methyl 3-formylbenzoate, Na(OAc)₃BH, 4A molsieves, DCE, rt, 16h, 60%; b) methyl 3-bromomethylbenzoate, NaOCH₃, MeOH, Ar(g), -20°C to rt, 16h, 75%; c) methyl 3-bromomethylbenzoate, NaH 55% dispersion in mineral oil, DMF, rt, 16h, 32%; d) 25% NH₃ (aq), MeOH, μ W, 130°C, 3h; e) TFA, DCM, rt, 1h.

NNMT crystal structure (Fig. 2), the active site Tyr₂₀ residue coordinates both the nicotinamide moiety and the carbonyl of the AdoMet amino acid group.

To separately assess the role of the AdoMet amino acid group, bisubstrate inhibitors comprised of a nicotinamide mimic linked to a carboxylic acid or amino acid moiety were next prepared (Schemes 3–5). A range of different linkers was used in preparing these conjugates. Based on the transition state model, the spacing between the carboxylic acid unit and the aromatic ring of the nicotinamide unit is 6 atoms. We therefore prepared analogues containing 5-, 6-, and 7-atom linkers to investigate the optimal spacing for inhibitor design. To examine the binding requirements for interaction with the NNMT amino acid binding pocket, compounds bearing a terminal carboxylic acid, a primary amide, or an amino acid moiety were prepared. In preparing compounds **18–25** (Scheme 3A) a Sonogashira cross-coupling reaction was used to couple fragment pairs. Conveniently, we found that the cross-coupling reaction did not require protecting groups on either the amide or carboxyl functionalities which significantly simplified access to the target compounds. Furthermore, formation of the intermediate alkyne provided the opportunity to also investigate the effect of altered rigidity and geometry in the linkers. Reduction of the alkyne intermediates **12–17** using standard hydrogenation conditions led to clean formation of the fully saturated species **18–23**. Partial reduction was also investigated to provide both the *E*- and *Z*-alkene variants derived from the 6-carbon spaced compound **14**. To this end the *Z*-alkene **24** was prepared using the Lindlar catalyst while the *E*-alkene **25** was accessed using Cp*RuCl(cod) as a catalyst.¹⁸ In both cases, the *E/Z* ratio was greater than 95% and the desired products were well separable from both the alkyne starting material and any over-reduced alkane side-product. Compounds **27** and **28** were synthesized in similar fashion starting from Boc-protected homo-propargylglycine (Scheme 3B). The most active compounds in this series were the 6- and 7-carbon spaced compounds **14**, **16**, **20**, and **22** containing a terminal carboxylic acid (see Table 1). The exception is compound **23**, which contains a terminal amide. The lack of inhibitory activity for

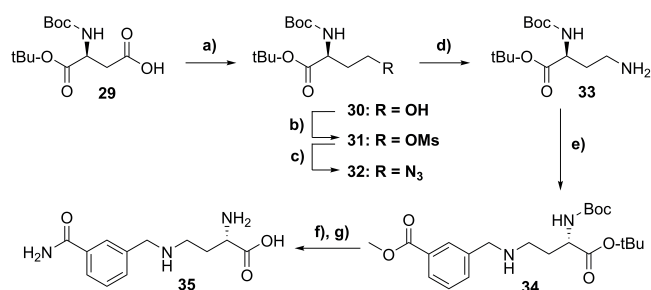


Scheme 3. A) Synthesis of alkynes **12–17** via Sonogashira cross-coupling and subsequent reduction of the alkynes to alkenes **24** and **25** and alkanes **18–23**. B) Synthesis of alkyne **27** and its corresponding alkane **28**. *Reagents and conditions:* a) Pd(PPh₃)₂Cl₂, CuI, Et₃N, THF, 50°C, 4h, 27–89%; b) 10% Pd/C, H₂, MeOH, rt, 1h, 62–88%; c) Lindlar catalyst, H₂, MeOH, rt, 3h, 85%; d) Cp*RuCl(cod), H₂, DCM, rt, 16h, 60%; e) TFA, DCM, rt, 1h.

compounds **24–28** is rather surprising given their structural similarity to the active analogues.

In addition to the carbon linked compounds, we also prepared analogues containing hetero-atom spacers consisting of either nitrogen or sulphur (Schemes 4 and 5). Synthesis of the amine linked conjugate **35** was achieved via a reductive amination approach similar to that used for the synthesis of compound **9**. As illustrated in Scheme 4, the protected diaminobutyric acid building block **33** was coupled with methyl-3-formylbenzoate to yield intermediate **34** which was subsequently deprotected and amidated using the same microwave-assisted procedure described for the preparation of **9–11**. Preparation of the thioether spaced conjugate **41** (Scheme 5) required access to thioacetate **38** which was generated via an unusual but effective method using potassium thioacetate absorbed on silica gel.¹⁹ Deprotection of **38** by treatment with potassium carbonate was followed by coupling with bromide **36**²⁰ to form protected thioether **40**. Final compound **41** was obtained using the general procedures for deprotection and amidation.

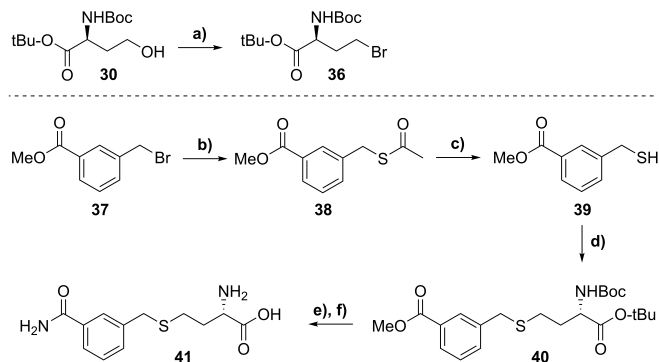
To complete the library of bisubstrate-based inhibitors, the ternary compound **45** was also designed with the aim of simultaneously interacting with all three binding pockets of the NNMT active site (Scheme 6). To this end the previously prepared compound **6** (Scheme 2) was coupled to aldehyde **43**²¹ via reductive amination to give **44**, which was subsequently deprotected and amidated to yield **45**. In performing the NNMT inhibition assays a number of compounds known to inhibit the enzyme were also evaluated



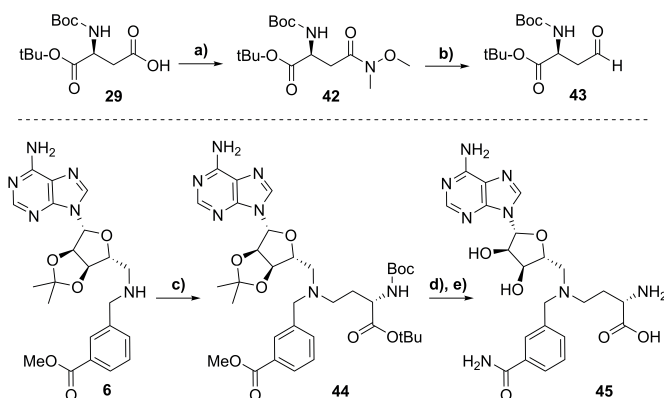
Scheme 4. Synthesis of compound **35**. Reagents and conditions: a) 1. Ethyl chloroformate, N-methyl morpholine, THF, -10°C to -5°C , 15 min 2. NaBH_4 , water, 5°C , 3.5 h, 95%; b) MsCl , DiPEA , DCE , 0°C , 30 min, 66%; c) NaN_3 , DMF , rt, 16h, 72%; d) Pd/C , H_2 , EtOH , rt, 1h, 89%; e) methyl-3-formylbenzoate, $\text{Na}(\text{OAc})_3\text{BH}$, DCE , rt, 16h, 37%; f) TFA , DCM , rt, 1h; g) 25% NH_3 (aq), MeOH , μW , 130°C , 3h.

as reference compounds (Fig. 3). As summarized in Table 1, the general methyltransferase inhibitors AdoHcy (IC_{50} 75.4 ± 6.3 μM) and sinefungin (17.0 ± 3.4 μM) display clear inhibition of NNMT. Also included was the NNMT product inhibitor MNA (IC_{50} 24.6 ± 3.2 μM). Given that MNA itself is the analyte detected in the standard NNMT activity assay, the alternative NNMT substrate 4-methyl-nicotinamide (4MeNA) was in this case used. In this regard, we also evaluated 4-methyl- N' -methyl-nicotinamide (4MeMNA) as an additional NNMT product inhibitor (IC_{50} 95.9 ± 14.1 μM), using the standard assay conditions. We also studied the ability of the polyaromatic compound norharmane to inhibit NNMT. Previous studies revealed norharmane to be a weak substrate for NNMT with a K_M value of 90 μM , significantly lower than that of the native substrate NA (K_M 200 μM), but with a very low turnover rate.²² We were therefore interested in assessing whether such a strong binding, slowly converted NNMT substrate might in fact act as an inhibitor by occupying the NNMT active site.^{16,22} Indeed, it was found that norharmane inhibits NNMT with an IC_{50} of 115.3 ± 20.6 μM . The low K_M is offset by a diminished turnover rate giving norharmane the ability to occupy the NNMT active site and effectively block the methylation of nicotineamide.

Among the newly synthesized compounds tested, only those containing structural elements derived from both the nicotineamide unit and the amino acid moiety of AdoMet resulted in appreciable inhibition (>50% at 250 μM).



Scheme 5. Synthesis of compound **41**. Reagents and conditions: a) CBr_4 , PPh_3 , DCM , 27%; b) $\text{SiO}_2\text{:AcSK}$, toluene, rt, 1h, 91%; c) K_2CO_3 , MeOH , rt, 2h, 93%; d) **36**, K_2CO_3 , DMF , rt, 2h, 60%; e) TFA , DCM , rt, 1h; f) 25% NH_3 (aq), MeOH , μW , 130°C , 3h.



Scheme 6. Synthesis of compound **45**. Reagents and conditions: a) O,N -dimethyl-hydroxylamine.HCl, BOP , Et_3N , DCM , rt, 2h, 90%; b) DiBAL-H in hexanes (1M), THF , -78°C , 87%; c) **43**, $\text{Na}(\text{OAc})_3\text{BH}$, DCE , rt, 16h, 53%; d) 25% NH_3 (aq), MeOH , μW , 130°C , 3h; e) TFA , DCM , rt, 1h.

Interestingly, compounds **9–11** wherein the adenosine moiety was linked to a benzamide unit designed to mimic nicotineamide, showed no significant inhibition. Rather, linking of the benzamide unit to a simple carboxylate as in **14**, **16**, **20**, **22**, and **23** (amide not carboxylate) gave much better inhibition and provide key insights into the preferred spacing and rigidity of the linker used to connect the respective bisubstrate-mimicking functionalities. The results indicate that the optimal spacing is 6–7 atoms. Furthermore, trivalent compound **45**, designed to bind in all three NNMT binding

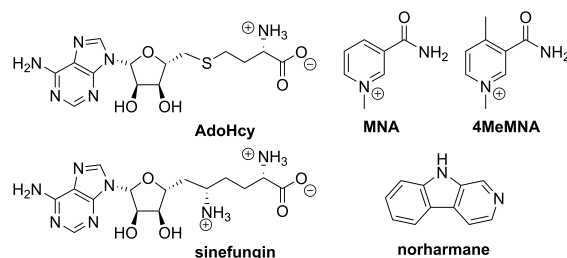


Figure 3. Structures of reference compounds AdoHcy, sinefungin, N' -methyl-nicotinamide (MNA) and 4-methyl- N' -methyl-nicotinamide (4MeMNA) and alternative NNMT substrate norharmane.

Table 1. IC_{50} values for NNMT inhibitors evaluated.^a

Compound	IC_{50} in μM	Compound	IC_{50} in μM
sinefungin	17.0 ± 3.4	18	> 250
MNA	24.6 ± 3.2	19	> 250
AdoHcy	75.4 ± 6.3	20	69.0 ± 14.8
4MeMNA	95.9 ± 14.1	21	> 250
norharmane	115.3 ± 20.6	22	148.1 ± 36.3
2a–i	> 250	23	30.8 ± 3.6
9	> 250	24	> 250
10	> 250	25	> 250
11	> 250	26	> 250
12	> 250	27	> 250
13	> 250	28	> 250
14	189.7 ± 30.0	35	> 250
15	> 250	41	> 250
16	57.8 ± 4.2	45	29.2 ± 4.0
17	> 250		

^a Assays performed in triplicate on at least six different inhibitor concentrations. Standard errors reported.

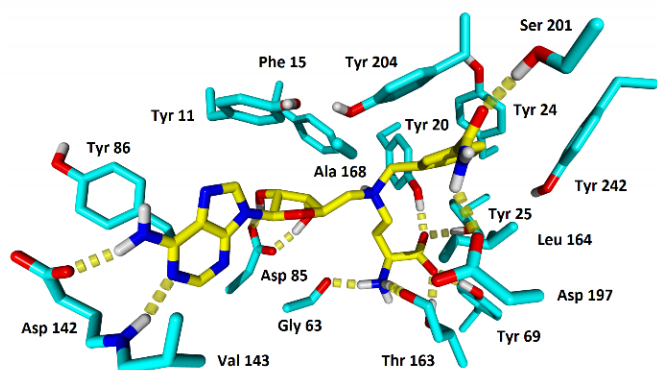


Figure 4. Proposed hydrogen bond network after modelling and minimization of compound **45** (yellow) in the NNMT active site (aliphatic hydrogens omitted for clarity).

pockets, inhibits NNMT with an IC_{50} of $29.2 \pm 4.0 \mu M$, similar to the inhibitory activity of reference inhibitors sinefungin and MNA.

Modelling studies performed with compound **45** show interactions in the enzyme's active site similar to those expected based upon the transition state model (Fig. 4). The docking analysis reveals that the spacing of the compound allows binding in all three binding pockets as hypothesized. Although the NA pyridine ring is replaced by a benzene ring, the pi-pi-stacking with tyrosine residue Y204 remains, which appears to be sufficient to compensate for the loss of interaction with the nitrogen in the pyridine ring of NA. All other interactions that were found in the crystal structure of NNMT in complex with nicotinamide and AdoHcy, shown in Figure 2, are visible in the model as represented in Figure 4 as well.

Conclusions

We here describe the first systematic bisubstrate approach towards the discovery of inhibitors of NNMT. While a number of partial bisubstrate analogues were explored, our results indicate that the best mimic of the NNMT transition state is the trivalent compound **45**. The absence of inhibitory activity for the alkylated nicotinamides **2a-i** and adenosine-nicotinamide conjugates **9-11** are somewhat surprising. While MNA shows an IC_{50} of $24.6 \mu M$, *N*'-ethylnicotinamide has an IC_{50} of $>250 \mu M$ as do analogues bearing larger alkyl groups. Furthermore, the simplified bisubstrate approach that we previously applied in generating potent inhibitors of the protein arginine methyltransferases¹⁷ was not successful here as compounds **9-11** displayed essentially no NNMT inhibition. While the set of compounds here described is not exhaustive, the results do suggest that binding in at least the nicotinamide and amino acid binding pockets is necessary for significant inhibition of the enzyme. An additional finding of interest is the inhibitory activity of norharmane, a known NNMT substrate with a low turnover rate. In this regard, incorporation of a norharmane mimic in future inhibitor designs may prove useful. Future plans are aimed at further optimizing compound **45** in an attempt to enhance inhibition.

To this end it is beneficial that the three main structural elements found in **45** can be modified independently and with relative ease. Once more potent NNMT inhibitors have been identified, their selectivity and activity in cell-based assays will be assessed. These findings will be reported in due course.

Experimental

General methods

All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. For compound characterization 1H NMR spectra were recorded at 400 MHz with chemical shifts reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS), H_2O (δ 4.79), methanol (δ 3.31) or DMSO (δ 2.50). 1H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet and m, multiplet), coupling constant (J) in hertz (Hz) and the number of protons. Where appropriate, the multiplicity is preceded by br, indicating that the signal was broad. ^{13}C NMR spectra were recorded at 101 MHz with chemical shifts reported relative to $CDCl_3$ (δ 77.16), methanol (δ 49.00) or DMSO (δ 39.52). The ^{13}C NMR spectra of the compounds recorded in D_2O could not be referenced. High-resolution mass spectrometry (HRMS) analysis was performed using a Q-TOF instrument. Compounds **3-5**¹⁷, **30-33**²³, **36**²⁰ and **42-43**²⁴ were synthesized according to previously described procedures. All known compounds prepared had NMR spectra and mass spectra consistent with the assigned structures. Purity was confirmed to be $\geq 95\%$ by analytical RP-HPLC using a Phenomenex Kinetex C18 column (5 μm , 250×4.6 mm) eluted with a water–acetonitrile gradient moving from 0% to 50% CH_3CN (0.1% TFA) over 30 minutes at a flow rate of 1.0 ml min^{-1} with UV detection at 214 nm and 254 nm.

General procedure alkylated nicotinamides

Nicotinamide (500 mg, 1.0 eq, 4.0 mmol) was dissolved in 10 mL dry CH_3CN , followed by the addition of R-I or R-Br (2.2 eq, 9 mmol). The tube was then sealed and reacted in a microwave for 90 minutes at $140^\circ C$. If necessary, the product was precipitated with dry diethyl ether. The precipitated product was filtered off and washed with dry diethyl ether.

1-Ethyl-3-carbamoyl-pyridinium iodide **2a**

Synthesized according to the general procedure (92% yield). 1H NMR (400 MHz, D_2O) δ 9.38 (s, 1H), 9.09 (d, J = 6.1 Hz, 1H), 8.92 (d, J = 8.2 Hz, 1H), 8.24 (t, J = 7.2 Hz, 1H), 4.78 (d, J = 7.4 Hz, 2H), 1.71 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, D_2O) δ 165.9, 146.2, 144.0, 143.8, 133.9, 128.4, 58.0, 15.6. HRMS (ESI): calculated for $C_8H_{11}N_2O^+$ 151.0866, found 151.0867.

1-Propyl-3-carbamoyl-pyridinium bromide **2b**

Synthesized according to the general procedure (697 mg, 71%). 1H NMR (400 MHz, D_2O) δ 9.36 (s, 1H), 9.07 (d, J = 6.0 Hz, 1H), 8.93 (d, J = 8.1 Hz, 1H), 8.24 (t, J = 7.2 Hz, 1H), 4.70 (t, J = 7.3 Hz, 2H), 2.18 – 2.04 (m, 2H), 1.00 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, D_2O) δ 165.84, 146.47, 144.20, 143.82, 133.83,

128.33, 63.86, 24.12, 9.63. HRMS (ESI): calculated for $C_9H_{13}N_2O^+$ 165.1022, found 165.1020.

1-Butyl-3-carbamoyl-pyridinium bromide 2c

Synthesized according to the general procedure (671 mg, 63%). 1H NMR (400 MHz, D_2O) δ 9.35 (s, 1H), 9.07 (d, J = 6.1 Hz, 1H), 8.93 (d, J = 8.2 Hz, 1H), 8.23 (t, J = 7.2 Hz, 1H), 4.73 (t, J = 7.5 Hz, 2H), 2.12 – 2.00 (m, 2H), 1.49 – 1.34 (m, 2H), 0.98 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, D_2O) δ 165.85, 146.43, 144.18, 143.75, 133.84, 128.30, 62.30, 32.49, 18.68, 12.60. HRMS (ESI): calculated for $C_{10}H_{15}N_2O^+$ 179.1179, found 179.1175.

1-Allyl-3-carbamoyl-pyridinium bromide 2d

Synthesized according to the general procedure (800 mg, 80%). 1H NMR (400 MHz, DMSO- d_6) δ 9.53 (s, 1H), 9.22 (d, J = 5.5 Hz, 1H), 9.02 (d, J = 7.9 Hz, 1H), 8.65 (s, 1H), 8.31 (t, J = 6.8 Hz, 1H), 8.18 (s, 1H), 6.34 – 6.13 (m, 1H), 5.58 – 5.43 (m, 2H), 5.38 (d, J = 5.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 162.7, 146.4, 144.8, 143.7, 133.7, 131.4, 128.0, 122.4, 62.6. HRMS (ESI): calculated for $C_9H_{11}N_2O^+$ 163.0866, found 163.0863.

1-Isopropyl-3-carbamoyl-pyridinium iodide 2e

Synthesized according to the general procedure (534 mg, 45%). 1H NMR (400 MHz, D_2O) δ 9.37 (s, 1H), 9.14 (d, J = 5.9 Hz, 1H), 8.91 (d, J = 7.8 Hz, 1H), 8.23 (t, J = 7.0 Hz, 1H), 5.19 – 5.07 (m, 1H), 1.76 (s, 3H), 1.74 (s, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 162.8, 144.6, 143.6, 142.9, 134.0, 128.1, 64.8, 22.3. HRMS (ESI): calculated for $C_9H_{13}N_2O^+$ 165.1022, found 165.1016.

1-Cyclopropylmethyl-3-carbamoyl-pyridinium bromide 2f

Synthesized according to the general procedure (335 mg, 63%). 1H NMR (400 MHz, DMSO- d_6) δ 9.61 (s, 1H), 9.32 (d, J = 5.0 Hz, 1H), 8.98 (d, J = 7.6 Hz, 1H), 8.63 (s, 1H), 8.28 (t, J = 6.5 Hz, 1H), 8.16 (s, 1H), 4.59 (d, J = 7.0 Hz, 2H), 1.94 – 1.23 (m, 1H), 0.61 (s, 2H), 0.60 (s, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 162.8, 146.1, 144.4, 143.6, 133.7, 127.9, 64.9, 12.0, 4.1. HRMS (ESI): calculated for $C_9H_{11}N_2O^+$ 177.1022, found 177.1026.

1-Cyclobutylmethyl-3-carbamoyl-pyridinium bromide 2g

Synthesized according to the general procedure (510 mg, 47%). 1H NMR (400 MHz, D_2O) δ 9.28 (s, 1H), 9.01 (d, J = 6.1 Hz, 1H), 8.92 (d, J = 8.0 Hz, 1H), 8.21 (t, J = 6.8 Hz, 1H), 4.75 (d, J = 7.6 Hz, 2H), 3.01 (dt, J = 15.0, 7.7 Hz, 1H), 2.17 – 2.05 (m, 3H), 2.01 – 1.86 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ 165.8, 146.2, 143.7, 141.9, 133.8, 128.3, 66.4, 35.8, 17.4. HRMS (ESI): calculated for $C_{11}H_{15}N_2O^+$ 191.1179, found 191.1181.

1-Cyclohexylmethyl-3-carbamoyl-pyridinium bromide 2h

Synthesized according to the general procedure (342 mg, 29%). 1H NMR (400 MHz, D_2O) δ 9.29 (s, 1H), 9.01 (d, J = 5.2 Hz, 1H), 8.93 (d, J = 8.3 Hz, 1H), 8.23 (t, J = 7.3 Hz, 1H), 4.57 (d, J = 7.3 Hz, 2H), 2.12 – 1.98 (m, 1H), 1.83 – 1.54 (m, 4H), 1.32 – 1.04 (m, 6H). ^{13}C NMR (101 MHz, D_2O) δ 165.8, 146.6, 143.8, 141.8, 133.7, 128.2, 67.9, 39.0, 29.2, 25.4, 24.9. HRMS (ESI): calculated for $C_{13}H_{19}N_2O^+$ 219.1492, found 219.1499.

1-Benzyl-3-carbamoyl-pyridinium bromide 2i

Synthesized according to the general procedure (1063 mg, 89%). 1H NMR (400 MHz, DMSO- d_6) δ 9.73 (s, 1H), 9.38 (d, J = 6.0 Hz, 1H), 9.01 (d, J = 8.1 Hz, 1H), 8.66 (s, 1H), 8.30 (t, J = 7.1 Hz, 1H), 8.20 (s, 1H), 7.64 – 7.58 (m, 2H), 7.47 – 7.38 (m, 3H), 5.98 (s, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 162.7, 146.4, 144.8, 143.9, 134.0, 134.0, 129.4, 129.2, 129.1, 128.3, 63.4.

HRMS (ESI): calculated for $C_{13}H_{13}N_2O^+$ 213.1022, found 213.1022.

Methyl 3-((((3aR,4R,6R,6aR)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]-dioxol-4-yl)methyl)-amino)methyl)benzoate (6)

Amine **3** (500 mg, 1.63 mmol, 1.05 eq) was dissolved in 35 mL dry DCE and methyl 3-formylbenzoate (255 mg, 1.55 mmol, 1.0 eq) was added along with crushed 4 Å molsieves. The mixture was stirred for 5 hours at room temperature under N_2 atmosphere before $Na(OAc)_3BH$ (461 mg, 2.18 mmol, 1.4 eq) was added in small portions. The mixture was stirred overnight at room temperature under N_2 atmosphere. Conversion was monitored by TLC (DCM:MeOH 8:2). The solvent was evaporated, redissolved in 150 mL DCE and filtered. The organic layer was washed with saturated $NaHCO_3$ and the aqueous layer extracted with DCM. The organic layer was dried with Na_2SO_4 , filtered and concentrated yielding **6** (537 mg, 75%). 1H NMR (400 MHz, $CDCl_3$): δ 8.12 (s, 1H), 7.96 (s, 1H), 7.92 (d, J = 7.7 Hz, 1H), 7.87 (s, 1H), 7.49 (d, J = 7.5 Hz, 1H), 7.37 (t, J = 7.6 Hz, 1H), 6.03 – 5.89 (m, 3H), 5.49 (dd, J = 6.3, 3.3 Hz, 1H), 5.08 (dd, J = 6.3, 3.0 Hz, 1H), 4.44 – 4.36 (m, 1H), 3.90 (s, 3H), 3.88 – 3.78 (m, 2H), 3.00 – 2.82 (m, 2H), 2.35 (s, 1H), 1.62 (s, 3H), 1.39 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.2, 155.8, 153.2, 149.5, 140.6, 140.1, 132.7, 130.4, 129.3, 128.6, 128.4, 120.6, 114.6, 91.2, 85.6, 83.4, 82.4, 53.5, 52.2, 50.8, 27.5, 25.5.

3-((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)amino)-methyl)benzamide (9)

Compound **6** (128 mg, 0.27 mmol) was dissolved in 1 mL MeOH and 1 mL 25% NH_3 (aq) and crimp sealed. The mixture was reacted for 4 hours at 130°C in the microwave. The mixture was concentrated and redissolved in 7 mL TFA:H₂O (5:2). After 45 minutes at room temperature, the mixture was concentrated and purified by preparative HPLC. 1H NMR (D_2O): δ 8.39 (s, 1H), 8.17 (s, 1H), 7.83 (d, J = 7.9 Hz, 1H), 7.78 (s, 1H), 7.65 (d, J = 7.8 Hz, 1H), 7.55 (t, J = 7.7 Hz, 1H), 6.15 (d, J = 4.2 Hz, 1H), 4.90 – 4.77 (m, 1H+H₂O), 4.54 (t, J = 5.3 Hz, 1H), 4.49 – 4.32 (m, 4H), 3.64 – 3.37 (m, 3H). ^{13}C NMR (101 MHz, D_2O) δ 171.5, 148.1, 147.7, 133.7, 133.3, 130.8, 129.5, 128.9, 128.3, 119.2, 90.1, 79.8, 73.3, 71.5, 50.4, 47.9. HRMS: calculated for $C_{18}H_{21}N_7O_4$ [M+H]⁺ 400.1728, found 400.1758.

Methyl 3-((((3aR,4R,6R,6aR)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]-dioxol-4-yl)methyl)thio)methyl)benzoate (7)

Thioacetate **4** (546 mg, 1.5 mmol, 1.0 eq) was dissolved in 30 mL dry deoxygenated methanol and methyl-3-bromomethylbenzoate (514 mg, 2.2 mmol, 1.5 eq) was added. The mixture was cooled to -20°C and freshly prepared sodium methoxide (from 82 mg Na(s) in 3 mL MeOH) was added. The mixture was stirred overnight at room temperature under N_2 atmosphere. The solvent was evaporated, redissolved in chloroform and washed with water. The organic layer was dried, concentrated and purified by column chromatography (2.5% MeOH in DCM) yielding **7** (450 mg, 64%) 1H NMR (400 MHz, $CDCl_3$): δ 8.27 (s, 1H), 7.94 – 7.84 (m, 3H), 7.39 (d, J = 7.6 Hz, 1H), 7.31 (t, J = 7.6 Hz, 1H), 6.07 – 6.00 (m, 3H), 5.44 (dd, J = 6.4, 1.9 Hz, 1H), 4.99

(dd, $J = 6.3, 3.4$ Hz, 1H), 4.33 (td, $J = 6.6, 3.4$ Hz, 1H), 3.89 (s, 3H), 3.72 (s, 2H), 2.71 (ddd, $J = 43.7, 13.7, 6.7$ Hz, 2H), 1.59 (s, 3H), 1.37 (s, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ 166.9, 155.8, 153.2, 149.3, 140.0, 138.4, 133.4, 130.6, 130.0, 128.7, 128.5, 120.4, 114.6, 90.8, 86.8, 84.1, 83.9, 52.3, 36.3, 33.5, 27.2, 25.5.

3-((((2S,3S,4R,5R)-5-(6-Amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)thio)methyl)benzamide (10)

Compound **7** (100 mg, 0.21 mmol) was dissolved in 1 mL MeOH and 1 mL 25% NH_3 (aq) and crimp sealed. The mixture was reacted for 4 hours at 130°C in the microwave. The mixture was concentrated and redissolved in 7 mL TFA:H₂O (5:2). After 45 minutes at room temperature, the mixture was concentrated and purified by preparative HPLC. ^1H NMR (400 MHz, CD_3OD): δ 8.43 (s, 1H), 8.33 (s, 1H), 7.71 (s, 1H), 7.68 (d, $J = 7.7$ Hz, 1H), 7.47 (d, $J = 7.6$ Hz, 1H), 7.40 (t, $J = 7.6$ Hz, 1H), 6.05 (d, $J = 4.8$ Hz, 1H), 4.78 (t, $J = 5.1$ Hz, 1H), 4.34 (t, $J = 5.1$ Hz, 1H), 4.24 (dt, $J = 6.5, 4.8$ Hz, 1H), 3.82 (d, $J = 2.2$ Hz, 2H), 2.92 (dd, $J = 14.2, 4.8$ Hz, 1H), 2.82 (dd, $J = 14.2, 6.7$ Hz, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 172.7, 149.5, 140.1, 134.2, 133.8, 129.9, 128.8, 127.1, 120.1, 89.9, 85.3, 74.6, 73.6, 67.7, 36.8, 34.0. HRMS: calculated for $\text{C}_{18}\text{H}_{21}\text{N}_6\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 417.1340, found 417.1366.

Methyl 3-((((3aR,4R,6R,6aR)-6-(6-(N-benzoylbenzamido)-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro-[3,4-d][1,3]dioxol-4-yl)methoxy)methyl)benzoate (8)

The protected adenosine **5** (1.0 g, 1.94 mmol, 1.0 eq) was added to a suspension of sodium hydride as dispersion in mineral oil (103 mg, 2.14 mmol, 1.1 eq) dissolved in 15 mL dry DMF. After 30 minutes a solution of methyl-3-bromomethylbenzoate (580 mg, 2.52 mmol, 1.3 eq) in DMF (2 mL) was added slowly and the mixture was stirred overnight at room temperature. The reaction was quenched with saturated NH_4Cl and the mixture was concentrated. The residue was redissolved in EtOAc and washed with saturated NH_4Cl . The aqueous phase was extracted with EtOAc and the combined organic layers were dried over sodium sulfate, concentrated and purified by column chromatography (2.5% MeOH in DCM) yielding **8** (450 mg, 64%). ^1H NMR (400 MHz, CDCl_3): δ 8.49 (s, 1H), 8.09 (s, 1H), 7.96 (s, 1H), 7.92 (d, $J = 7.5$ Hz, 2H), 7.84 (d, $J = 7.7$ Hz, 1H), 7.72 (d, $J = 7.8$ Hz, 1H), 7.52 (t, $J = 7.4$ Hz, 1H), 7.45 (d, $J = 7.5$ Hz, 2H), 7.36 (t, $J = 7.8$ Hz, 2H), 7.31 (t, $J = 7.7$ Hz, 1H), 7.28 – 7.21 (m, 1H), 7.14 (t, $J = 7.6$ Hz, 2H), 6.06 (d, $J = 2.3$ Hz, 1H), 5.62 (d, $J = 2.5$ Hz, 2H), 5.45 (dd, $J = 6.4, 2.3$ Hz, 1H), 5.10 (dd, $J = 6.3, 3.3$ Hz, 1H), 4.60 – 4.49 (m, 2H), 4.43 (dd, $J = 11.5, 5.3$ Hz, 1H), 3.85 (s, 3H), 1.61 (s, 3H), 1.38 (s, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ 172.1, 167.0, 166.1, 154.3, 152.2, 152.0, 142.7, 138.0, 135.9, 133.4, 132.6, 131.1, 130.3, 129.7, 129.6, 129.4, 129.3, 128.9, 128.7, 128.6, 128.6, 128.5, 128.0, 127.0, 115.1, 91.1, 84.7, 83.9, 81.4, 64.0, 52.1, 51.2, 27.3, 25.5.

3-((((2S,3S,4R,5R)-5-(6-Amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)methyl)benzamide (11)

Compound **8** (100 mg, 0.21 mmol) was dissolved in 1 mL MeOH and 1 mL 25% NH_3 (aq) and crimp sealed. The mixture was reacted for 4 hours at 130°C in the microwave. The mixture was concentrated and redissolved in 7 mL TFA:H₂O

(5:2). After 45 minutes at room temperature, the mixture was concentrated and purified by preparative HPLC. ^1H NMR (400 MHz, D_2O): δ 8.47 (s, 1H), 8.36 (s, 1H), 7.79 (s, 1H), 7.74 (d, $J = 7.5$ Hz, 1H), 7.61 (d, $J = 7.5$ Hz, 1H), 7.50 (t, $J = 7.7$ Hz, 1H), 6.13 (d, $J = 5.2$ Hz, 1H), 5.00 – 4.83 (m, 2H), 4.76 (t, $J = 5.3$ Hz, 1H), 4.44 (t, $J = 5.1$ Hz, 1H), 4.29 (d, $J = 30.9$ Hz, 1H), 3.97 – 3.79 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ ^{13}C NMR (101 MHz, d_2o) δ 172.4, 133.1, 131.1, 129.2, 126.8, 126.1, 117.7, 114.8, 88.5, 85.6, 74.1, 70.2, 61.1. HRMS: calculated for $\text{C}_{18}\text{H}_{21}\text{N}_6\text{O}_5$ $[\text{M}+\text{Na}]^+$ 423.1387, found 423.1411.

5-(3-Carbamoylphenyl)pent-4-ynoic acid (12)

To a suspension of 4-pentynoic acid (122 mg, 1.24 mmol), 3-iodo-*N*-tritylbenzamide (490 mg, 1.0 mmol) and TEA (5 mL), $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ (14 mg, 0.02 mmol) was added and stirred at room temperature for 5 minutes under N_2 environment. After addition of THF (2 mL) and $\text{Cu}(\text{I})\text{I}$ (8 mg, 0.04 mmol), the mixture was heated to 50°C for 4 hours. The solvent was evaporated *in vacuo*, and the crude product was purified by column chromatography (SiO_2 : DCM - 5% MeOH) affording the trityl-protected product (89 mg, 20%) as an orange powder. The intermediate was dissolved in DCM (2 mL) and a mixture of TFA/TIPS/H₂O (95:2.5:2.5, 5 mL) was added. After full deprotection as determined via TLC (DCM / 5% MeOH), the mixture was concentrated and purified by preparative HPLC to yield compound **12** as a white powder (21 mg, 50%). ^1H NMR (400 MHz, CD_3OD) δ 7.88 (s, 1H), 7.77 (d, $J = 7.8$ Hz, 1H), 7.53 (d, $J = 7.7$ Hz, 1H), 7.41 (t, $J = 7.8$ Hz, 1H), 2.72 (t, $J = 7.2$ Hz, 2H), 2.51 (t, $J = 7.3$ Hz, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 176.9, 171.5, 135.6, 135.3, 131.7, 129.6, 127.9, 125.5, 90.3, 81.3, 35.5, 16.4. HRMS: calculated for $\text{C}_{12}\text{H}_{11}\text{NO}_3$ $[\text{M}+\text{H}]^+$: 218.0812, found 218.0804.

5-(3-Carbamoylphenyl)pent-4-ynoic amide (13)

Following the procedure described for compound **12**, coupling 4-pentynoic amide (117 mg, 1.2 mmol) and 3-iodobenzamide (247 mg, 1.0 mmol) afforded compound **13** (169 mg, 78%) as a white powder. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 7.99 (s, 1H), 7.83 (s, 1H), 7.78 (d, $J = 7.7$ Hz, 1H), 7.46 (d, $J = 7.5$ Hz, 1H), 7.41 – 7.31 (m, 2H), 6.85 (s, 1H), 2.59 (t, $J = 7.3$ Hz, 2H), 2.32 (t, $J = 7.3$ Hz, 2H). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 172.76, 167.48, 134.99, 134.18, 130.59, 129.03, 127.47, 123.52, 91.10, 80.33, 34.47, 15.41. HRMS: calculated for $\text{C}_{12}\text{H}_{12}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}]^+$: 217.0972, found 217.0991.

6-(3-Carbamoylphenyl)hex-5-ynoic acid (14)

Following the procedure described for compound **12**, coupling of 5-hexynoic acid (137 mg, 1.22 mmol) and 3-iodo-*N*-tritylbenzamide (490 mg, 1.0 mmol) afforded the trityl-protected intermediate (232 mg, 49 %) as a peach colored powder. The deprotection and purification procedure on 0.2 mmol scale as described for compound **12** afforded final compound **14** (21 mg, 42%). ^1H NMR (400 MHz, CD_3OD) δ 7.86 (s, 1H), 7.76 (d, $J = 7.8, 1.7$ Hz, 1H), 7.51 (d, $J = 7.7, 1.6$ Hz, 1H), 7.38 (t, $J = 1.8$ Hz, 1H), 2.51 – 2.43 (m, 4H), 1.92 – 1.82 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 175.46, 170.13, 134.18, 133.79, 130.20, 128.17, 126.42, 124.21, 89.49, 79.97, 32.33, 23.68, 17.98. HRMS: calculated for $\text{C}_{13}\text{H}_{13}\text{NO}_3$ $[\text{M}+\text{H}]^+$: 232.0968, found 232.0974.

6-(3-Carbamoylphenyl)hex-5-ynoic amide (15)

Following the procedure described for compound **12**, coupling 5-hexynoic amide (133 mg, 1.2 mmol) and 3-iodobenzamide (247 mg, 1.0 mmol) afforded compound **15** (144 mg, 63%) as a white powder. ^1H NMR (400 MHz, DMSO- d_6) δ 7.99 (s, 1H), 7.85 (s, 1H), 7.78 (d, J = 7.8 Hz, 1H), 7.50 (d, J = 7.7 Hz, 1H), 7.41 – 7.36 (m, 2H), 7.28 (s, 1H), 6.73 (s, 1H), 2.41 (t, J = 7.0 Hz, 2H), 2.19 (t, J = 7.4 Hz, 2H), 1.76 – 1.68 (m, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 174.03, 167.49, 134.97, 134.22, 130.59, 129.02, 127.46, 123.61, 91.24, 80.74, 34.33, 24.57, 18.72. HRMS: calculated for $\text{C}_{13}\text{H}_{13}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}]^+$: 231.1128, found 231.1131.

7-(3-Carbamoylphenyl)hept-6-ynoic acid (**16**)

Following the procedure described for compound **12**, coupling 6-heptynoic acid (302.7 mg, 2.4 mmol) and 3-iodo-*N*-tritylbenzamide (980 mg, 2.0 mmol) afforded the trityl-protected intermediate (712 mg, 72 %) as a yellow oil. The deprotection and purification procedure on 0.2 mmol scale as described for compound **12** afforded final compound **16** (15 mg, 27% isolated yield). ^1H NMR (400 MHz, CD_3OD) δ 7.84 (s, 1H), 7.75 (d, J = 7.8 Hz, 1H), 7.51 (d, J = 7.6 Hz, 1H), 7.38 (t, J = 7.8 Hz, 1H), 2.44 (t, J = 6.9 Hz, 2H), 2.34 (t, J = 7.2 Hz, 2H), 1.80 – 1.72 (m, 2H), 1.66 – 1.58 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 177.5, 171.7, 135.6, 135.1, 131.6, 129.6, 127.7, 125.7, 91.6, 81.0, 34.4, 29.1, 25.3, 19.6. HRMS: calculated for $\text{C}_{14}\text{H}_{15}\text{NO}_3$ $[\text{M}+\text{Na}]^+$: 268.0944, found 268.0941.

7-(3-Carbamoylphenyl)hept-6-ynoic amide (**17**)

Following the procedure described for compound **12**, coupling 5-hexynoic amide (150 mg, 1.2 mmol) and 3-iodo-*N*-tritylbenzamide (247 mg, 0.5 mmol) afforded the trityl-protected intermediate (218 mg, 89 %) as a yellow powder. The deprotection and purification procedure on 0.2 mmol scale as described for compound **12** afforded final compound **17** (22 mg, 44% isolated yield). ^1H NMR (400 MHz, CD_3OD) δ 7.88 (s, 1H), 7.78 (d, J = 7.8 Hz, 1H), 7.53 (d, J = 7.7 Hz, 1H), 7.40 (t, J = 7.8 Hz, 1H), 2.47 (t, J = 6.9 Hz, 3H), 2.27 (t, J = 7.4 Hz, 2H), 1.85 – 1.76 (m, 3H), 1.69 – 1.61 (m, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 178.9, 171.6, 135.6, 135.2, 131.6, 129.6, 127.8, 125.8, 91.6, 81.1, 36.0, 29.3, 26.1, 19.7. HRMS: calculated for $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}]^+$: 245.1258, found 245.1185.

5-(3-Carbamoylphenyl)pentanoic acid (**18**)

To a solution of the trityl-protected intermediate of compound **12** (90 mg, 0.19 mmol) in MeOH (5 mL), a suspension of Pd/C (10 %, 20 mg, excess) in MeOH (5 mL) was added. The mixture was hydrogenated using an H_2 -balloon for 2 hours. The catalyst was removed by filtration over celite and the solvent was evaporated in *vacuo*, affording the trityl-protected alkane (56 mg, 62 %) as a white powder. The deprotection and purification procedure as described for compound **12** afforded final compound **18** (9 mg, 33% isolated yield). ^1H NMR (400 MHz, CD_3OD) δ 7.69 (s, 1H), 7.66 (d, J = 6.7 Hz, 1H), 7.38 – 7.31 (m, 2H), 2.67 (d, J = 7.0 Hz, 2H), 2.30 (d, J = 7.1 Hz, 2H), 1.70 – 1.59 (m, 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 177.5, 172.6, 144.0, 134.9, 133.1, 129.5, 128.6, 126.2, 36.4, 34.7, 31.9, 25.6. HRMS: calculated for $\text{C}_{12}\text{H}_{15}\text{NO}_3$ $[\text{M}+\text{H}]^+$: 222.1125, found 222.1137.

5-(3-Carbamoylphenyl)pentanoic amide (**19**)

Compound **13** (100 mg, 0.46 mmol) was reduced following the procedure described for compound **18** affording final

compound **19** (69 mg, 67%) as a white powder. ^1H NMR (400 MHz, CD_3OD) δ 7.71 (s, 1H), 7.68 (d, J = 6.8 Hz, 1H), 7.40 – 7.33 (m, 2H), 2.71 (t, J = 7.1 Hz, 2H), 2.24 (t, J = 7.2 Hz, 2H), 1.72 – 1.62 (m, 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 179.1, 172.6, 144.1, 135.0, 133.1, 129.5, 128.6, 126.2, 36.4, 36.3, 32.0, 26.4. HRMS: calculated for $\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}]^+$: 221.1285, found 221.1294.

6-(3-Carbamoylphenyl)hexanoic acid (**20**)

Compound **14** (100 mg, 0.21 mmol) was reduced following the procedure described for compound **18** affording the trityl-protected alkane (88 mg, 88 %) as a white powder. The deprotection and purification procedure as described for compound **12** afforded final compound **20** (15 mg, 29% isolated yield). ^1H NMR (400 MHz, CD_3OD) δ 7.71 (s, 1H), 7.67 (d, J = 6.8 Hz, 1H), 7.40 – 7.33 (m, 2H), 2.69 (t, J = 7.6 Hz, 2H), 2.28 (t, J = 7.4 Hz, 2H), 1.72 – 1.61 (m, 4H), 1.43 – 1.34 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 177.6, 172.7, 144.3, 134.9, 133.1, 129.5, 128.6, 126.1, 49.6, 49.4, 49.2, 49.0, 49.0, 48.8, 48.6, 48.4, 36.5, 34.8, 32.2, 29.7, 25.9. HRMS: calculated for $\text{C}_{13}\text{H}_{17}\text{NO}_3$ $[\text{M}+\text{H}]^+$: 236.1281, found 236.1290.

6-(3-Carbamoylphenyl)hexanoic amide (**21**)

Compound **15** (96 mg, 0.42 mmol) was reduced following the procedure described for compound **18** affording final compound **21** (21 mg, 22%) as a white powder. ^1H NMR (400 MHz, CD_3OD) δ 7.71 (s, 1H), 7.67 (d, J = 6.8 Hz, 1H), 7.39 – 7.33 (m, 2H), 2.69 (d, J = 7.6 Hz, 2H), 2.20 (d, J = 7.4 Hz, 2H), 1.73 – 1.61 (m, 4H), 1.43 – 1.33 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 179.2, 172.6, 144.3, 134.9, 133.1, 129.5, 128.7, 126.1, 36.5, 36.4, 32.2, 29.7, 26.6. HRMS: calculated for $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}]^+$: 235.1441, found 235.1450.

7-(3-Carbamoylphenyl)heptanoic acid (**22**)

Compound **16** (100 mg, 0.21 mmol) was reduced following the procedure described for compound **18** affording the trityl-protected alkane (80 mg, 79 %) as a white powder. The deprotection and purification procedure as described for compound **12** (0.1 mmol scale) afforded final compound **22** (7.4 mg, 30% isolated yield). ^1H NMR (400 MHz, CD_3OD) δ 7.71 (s, 1H), 7.68 (d, J = 6.5 Hz, 1H), 7.41 – 7.34 (m, 2H), 2.69 (t, J = 7.5 Hz, 2H), 2.28 (t, J = 7.3 Hz, 2H), 1.71 – 1.56 (m, 4H), 1.42 – 1.35 (m, 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 172.7, 144.5, 134.9, 133.1, 129.5, 128.6, 126.1, 36.6, 35.0, 32.4, 30.0, 29.9, 26.0. HRMS: calculated for $\text{C}_{14}\text{H}_{19}\text{NO}_3$ $[\text{M}+\text{H}]^+$: 250.1438, found 250.1439.

7-(3-Carbamoylphenyl)heptanoic amide (**23**)

Compound **17** (90 mg, 0.19 mmol) was reduced following the procedure described for compound **18** affording the trityl-protected alkane (69 mg, 76%) as a white powder. The deprotection and purification procedure as described for compound **12** afforded final compound **23** (12 mg, 36% isolated yield). ^1H NMR (400 MHz, DMSO- d_6) δ 7.92 (s, 1H), 7.71 – 7.64 (m, 2H), 7.37 – 7.26 (m, 3H), 7.21 (s, 1H), 6.66 (s, 1H), 2.60 (t, J = 7.5 Hz, 2H), 2.01 (t, J = 7.4 Hz, 2H), 1.63 – 1.52 (m, 2H), 1.51 – 1.42 (m, 2H), 1.34 – 1.20 (m, 4H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 174.3, 168.0, 142.3, 134.2, 131.1, 128.1, 127.4, 124.8, 35.1, 35.0, 30.8, 28.5, 28.4, 25.0. HRMS: calculated for $\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}]^+$: 249.1598, found 249.1603.

(Z)-6-(3-Carbamoylphenyl)hex-5-enoic acid (**24**)

Alkyne **20** (70 mg, 0.30 mmol) was selectively reduced using Lindlar catalyst (15 mg, 20% w/w) in MeOH (3.5 mL) under H₂ atmosphere at room temperature. After 3 hours, the reaction was complete as seen by NMR. The catalyst was filtered off over celite, the solvent evaporated and the crude product purified by preparative HPLC affording (Z)-alkene **24** (60 mg, 0.26 mmol, 85%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.96 (s, 1H), 7.77 – 7.70 (m, 2H), 7.46 – 7.40 (m, 2H), 7.35 (s, 1H), 6.47 (d, *J* = 11.7 Hz, 1H), 5.71 (dt, *J* = 11.7, 7.2 Hz, 1H), 2.31 (q, *J* = 6.8 Hz, 2H), 2.24 (t, *J* = 7.4 Hz, 2H), 1.65 (p, *J* = 7.5 Hz, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 167.8, 163.1, 137.0, 134.4, 132.7, 131.1, 128.6, 128.2, 127.6, 125.7, 33.1, 27.5, 24.6. HRMS: calculated for C₁₃H₁₅NO₃ [M+Na]⁺: 256.0944, found 256.0966.

(E)-6-(3-Carbamoylphenyl)hex-5-enoic acid (25)

Alkyne **20** (50 mg, 0.22 mmol) was selectively reduced using a Cp*RuCl(cod) catalyst (4.1 mg, 5 mol-%) in DCM (3 mL) under H₂ atmosphere at room temperature. After overnight stirring, NMR shows 60% conversion with >95% *E*-alkene. The catalyst was filtered off over celite, the solvent evaporated and the crude product purified by preparative HPLC affording (E)-alkene **25**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.97 (s, 1H), 7.90 (s, 1H), 7.71 (d, *J* = 7.0 Hz, 1H), 7.52 (d, *J* = 7.0 Hz, 1H), 7.44 – 7.26 (m, 2H), 6.51 – 6.29 (m, 2H), 2.38 – 2.10 (m, 4H), 1.77 – 1.60 (m, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 174.3, 167.8, 137.3, 134.5, 131.0, 129.5, 128.7, 128.5, 126.0, 124.7, 33.1, 31.8, 24.1. HRMS: calculated for C₁₃H₁₅NO₃ [M+H]⁺: 234.1125, found 234.1143.

(S)-2-((Tert-butoxycarbonyl)amino)-6-(3-carbamoylphenyl) hex-5-ynoic acid (26)

Following the procedure described for compound **12**, coupling Boc-L-homopropargylglycine (114 mg, 0.5 mmol) and 3-iodobenzamide (130 mg, 0.5 mmol) afforded compound **26** (140 mg, 81%) as a white powder. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.02 (s, 1H), 7.88 (s, 1H), 7.82 (d, *J* = 7.8 Hz, 1H), 7.52 (d, *J* = 7.6 Hz, 1H), 7.47 – 7.37 (m, 2H), 7.14 (d, *J* = 7.9 Hz, 1H), 4.11 – 3.98 (m, 1H), 2.57 – 2.43 (m, 2H), 2.02 – 1.77 (m, 2H), 1.38 (s, 9H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 173.8, 167.1, 155.6, 134.6, 133.8, 130.2, 128.6, 127.1, 123.0, 90.1, 80.5, 78.1, 52.7, 29.9, 28.2, 15.8.

(S)-2-Amino-6-(3-carbamoylphenyl)hex-5-ynoic acid (27)

Compound **26** (70 mg, 0.2 mmol) was deprotected in TFA/DCM (1:1, 4 mL) for 2 hours at room temperature. The mixture was concentrated and the product purified by preparative HPLC affording compound **27** as a white powder. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.34 (br s, 2H), 8.02 (s, 1H), 7.90 (s, 1H), 7.84 (d, *J* = 7.8 Hz, 1H), 7.55 (d, *J* = 7.7 Hz, 1H), 7.49 – 7.40 (m, 2H), 4.03 (t, *J* = 6.0 Hz, 1H), 2.74 – 2.53 (m, 2H), 2.19 – 1.97 (m, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 170.6, 167.0, 134.6, 133.8, 130.3, 128.6, 127.2, 122.8, 89.0, 81.0, 51.2, 29.2, 15.1. HRMS: calculated for C₁₃H₁₄N₂O₃ [M+H]⁺: 247.1077, found 247.1105.

(S)-2-Amino-6-(3-carbamoylphenyl)hexanoic acid (28)

Compound **26** (100 mg, 0.46 mmol) was reduced following the procedure described for compound **18**. The intermediate was deprotected in TFA/DCM (1:1, 4 mL) for 2 hours at room temperature. The mixture was concentrated and the product was purified by preparative HPLC affording compound **28** as a white powder. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.20 (s, 2H),

7.91 (s, 1H), 7.73 – 7.65 (m, 2H), 7.39 – 7.29 (m, 3H), 3.90 (t, *J* = 5.8 Hz, 1H), 2.62 (t, *J* = 7.6 Hz, 2H), 1.79 (tt, *J* = 14.0, 6.7 Hz, 2H), 1.61 (p, *J* = 7.5 Hz, 2H), 1.51 – 1.26 (m, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.1, 168.0, 141.9, 134.3, 131.2, 128.1, 127.4, 124.9, 51.9, 34.7, 30.3, 29.9, 24.0. HRMS: calculated for C₁₃H₁₈N₂O₃ [M+H]⁺: 251.1390, found 251.1414.

Methyl-(S)-3-(((4-(tert-butoxy)-3-((tert-butoxycarbonyl)amino)-4-oxobutyl)amino)methyl)benzoate (34)

To a solution of methyl 3-formylbenzoate (140 mg, 0.9 mmol) and compound **33** (220 mg, 0.8 mmol) in dry DCE (15 mL), crushed 4 Å molsieves were added. After 2 hours, Na(OAc)₃BH (254 mg, 1.2 mmol) was added over 5 minutes. The mixture was reacted overnight, quenched with saturated NaHCO₃, filtered and the aqueous phase extracted with DCM. The organic layers were combined, washed with Brine, dried and concentrated. Purification via column chromatography (gradient of 2.5 to 6% MeOH in DCM) afforded compound **34** (90 mg, 27 %) as a white powder. ¹H NMR (400 MHz, CDCl₃) δ 7.94 (s, 1H), 7.89 (d, *J* = 7.7 Hz, 1H), 7.52 (d, *J* = 7.4 Hz, 1H), 7.36 (t, *J* = 7.6 Hz, 1H), 5.65 (d, *J* = 7.4 Hz, 1H), 4.34 – 4.10 (m, 1H), 3.88 (s, 3H), 3.78 (s, 2H), 2.68 (t, *J* = 6.6 Hz, 2H), 2.06 – 1.69 (m, 2H), 1.40 (s, 18H). ¹³C NMR (101 MHz, CDCl₃) δ 171.9, 167.1, 155.7, 140.5, 132.9, 130.3, 129.3, 128.6, 128.4, 81.8, 79.5, 53.6, 52.8, 52.1, 45.4, 32.6, 28.4, 28.0.

(S)-2-Amino-4-((3-carbamoylbenzyl)amino)butanoic acid (35)

Compound **34** (80 mg, 0.19 mmol) was first deprotected in TFA:DCM (1:2) for 1 hour at room temperature. The mixture was concentrated, redissolved in 1 mL MeOH and 1 mL 25% NH₃ (aq) and crimp sealed. The mixture was reacted for 4 hours at 130°C in the microwave. The mixture was concentrated and purified by preparative HPLC affording compound **34** as a white powder. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.06 – 8.01 (m, 2H), 7.92 (d, *J* = 7.8 Hz, 1H), 7.62 (d, *J* = 7.7 Hz, 1H), 7.52 (t, *J* = 7.6 Hz, 1H), 7.46 (s, 1H), 4.23 (s, 2H), 4.12 – 3.96 (m, 1H), 3.24 – 2.96 (m, 2H), 2.26 – 2.01 (m, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 170.2, 167.4, 134.7, 132.6, 132.0, 129.5, 128.6, 127.7, 50.0, 49.7, 43.3, 26.5. HRMS: calculated for C₁₂H₁₇N₃O₃ [M+H]⁺: 252.1343, found 252.1360.

Methyl 3-((acetylthio)methyl)benzoate (38)

Following a procedure described in literature¹⁹, methyl-3-bromomethylbenzoate (1.15 g, 5.0 mmol) was reacted with silica-gel supported potassium thioacetate (7.5 g, 1 mmol/g, 7.5 mmol) in toluene (20 mL). After 1 hour at room temperature, the mixture was filtered and concentrated yielding compound **38** as a slightly yellow oil (1.01 g, 91%). The compound had NMR spectra and mass spectra consistent with the assigned structures²⁵.

Methyl 3-(mercaptomethyl)benzoate (39)

Following a procedure described in literature²⁶, compound **38** (224 mg, 1.0 mmol) was deacetylated using potassium carbonate (350 mg, 2.5 mmol) in methanol (15 mL). After stirring for 2 hours at room temperature, the reaction was quenched using 0.1 M HCl to pH 2.5, diluted with water and extracted with DCM. The organic phase was washed with Brine, dried and concentrated yielding compound **39** (170 mg,

93%). The compound had NMR spectra and mass spectra consistent with the assigned structures²⁷.

Methyl (S)-3-(((4-(tert-butoxy)-3-((tert-butoxycarbonyl)-amino)-4-oxobutyl)thio)methyl)benzoate (40)

To a solution of thiol **39** (170 mg, 0.9 mmol) and bromide **36** (270 mg, 0.8 mmol) in dry DMF (10 mL), potassium carbonate (350 mg, 2.5 mmol) was added. After 2 hours, at room temperature, TLC shows completion and the mixture was concentrated, redissolved in DCM and washed with water. The aqueous phase was extracted with DCM, the organic layers were combined, washed with Brine, dried and concentrated. Purification via column chromatography (hexanes/ EtOAc 8:1) afforded compound **40** (245 mg, 60 %) as a clear oil. ¹H NMR (400 MHz, CDCl₃) δ 7.94 (s, 1H), 7.88 (d, *J* = 7.8 Hz, 1H), 7.49 (d, *J* = 7.7 Hz, 1H), 7.35 (t, *J* = 7.7 Hz, 1H), 5.08 (d, *J* = 7.5 Hz, 1H), 4.23 – 4.17 (m, 1H), 3.88 (s, 3H), 3.71 (s, 2H), 2.45 – 2.35 (m, 2H), 2.08 – 1.74 (m, 2H), 1.39 (s, 18H). ¹³C NMR (101 MHz, CDCl₃) δ 171.3, 166.8, 155.3, 138.8, 133.4, 130.5, 129.9, 128.7, 128.4, 82.1, 79.7, 53.4, 52.2, 35.9, 32.8, 28.3, 28.0, 27.2.

(S)-2-Amino-4-((3-carbamoylbenzyl)thio)butanoic acid (41)

Compound **40** (100 mg, 0.23 mmol) was first deprotected in TFA:DCM (1:2) for 1 hour at room temperature. The mixture was concentrated, redissolved in 1 mL MeOH and 1 mL 25% NH₃ (aq) and crimp sealed. The mixture was reacted for 4 hours at 130°C in the microwave. The mixture was concentrated and purified by preparative HPLC affording compound **41** as a white powder. ¹H NMR (400 MHz, D₂O) δ 7.77 (s, 1H), 7.71 (d, *J* = 8.0 Hz, 1H), 7.60 (d, *J* = 7.8 Hz, 2H), 7.49 (t, *J* = 7.7 Hz, 1H), 4.09 (t, *J* = 6.4 Hz, 1H), 3.86 (s, 2H), 2.66 – 2.55 (m, 2H), 2.29 – 2.04 (m, 2H). ¹³C NMR (101 MHz, D₂O) δ 172.6, 171.9, 138.8, 133.0, 132.8, 129.1, 127.7, 126.2, 51.9, 34.5, 29.3, 25.8. HRMS: calculated for C₁₂H₁₆N₂O₃ [M+H]⁺: 269.0954, found 269.0955.

Methyl 3-((((3aR,4R,6R,6aR)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]-dioxol-4-yl)methyl)-((S)-4-(tert-butoxy)-3-((tert-butoxycarbonyl)amino)-4-oxobutyl)-amino)-methyl)benzoate (44)

To a solution of compound **6** (200 mg, 0.44 mmol) and aldehyde **43** (126 mg, 0.46 mmol) in dry DCE (10 mL), crushed 4 Å molsieves were added. After 2 hours, Na(OAc)₃BH (130 mg, 0.62 mmol) was added over 5 minutes. The mixture was reacted overnight, quenched with saturated NaHCO₃, filtered and the aqueous phase was extracted with DCM. The organic layers were combined, washed with Brine, dried and concentrated. Purification via column chromatography (gradient of 2.5 to 7.5% MeOH in DCM) afforded compound **44** (156 mg, 53 %) as a white powder. ¹H NMR (400 MHz, CDCl₃) δ 8.20 (s, 1H), 7.89–7.87 (m, 2H), 7.83 (br s, 1H), 7.49 (d, *J* = 7.7 Hz, 1H), 7.30 (t, *J* = 7.9 Hz, 1H), 6.02 (br s, 1H), 5.73 (br s, 1H), 5.41–5.36 (br m, 2H), 4.90 (dd, *J* = 3.5, 6.4 Hz, 2H), 4.38–4.34 (m, 1H), 4.17–4.16 (m, 1H), 3.91 (s, 3H), 3.70 (d, *J* = 13.7 Hz, 1H), 3.54 (dd, *J* = 13.7 Hz, 1H), 2.81–2.76 (m, 2H), 2.70–2.61 (m, 2H), 2.55–2.48 (m, 2H), 2.01–1.97 (m, 2H), 1.80–1.76 (m, 1H), 1.60 (s, 3H), 1.43 (s, 9H), 1.40 (s, 9H), 1.37 (s, 3H), 1.26 (br s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 171.7, 167.1, 155.6, 153.1, 149.3, 139.3, 133.6, 130.1, 130.0, 128.5, 128.4, 120.5, 114.5,

90.9, 85.5, 83.9, 83.5, 81.8, 79.6, 58.8, 55.8, 52.9, 52.2, 50.7, 29.7, 28.5, 28.1, 27.3, 25.5. HRMS: calculated for C₃₅H₄₉N₇O₉ [M+H]⁺ 712.3365, found 712.3371.

(S)-2-Amino-4-((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)(3-carbamoylbenzyl)-amino)butanoic acid (45)

Compound **44** (80 mg, 0.19 mmol) was first deprotected in TFA:DCM (1:2) for 1 hour at room temperature. The mixture was concentrated, redissolved in 1 mL MeOH and 1 mL 25% NH₃ (aq) and crimp sealed. The mixture was reacted for 4 hours at 130°C in the microwave. The mixture was concentrated and purified by preparative HPLC affording compound **45** as a white powder. ¹H NMR (400 MHz, D₂O): δ 8.27 (s, 1H), 8.18 (s, 1H), 7.77 – 7.57 (m, 3H), 7.42 (t, *J* = 7.7 Hz, 1H), 6.10 (s, 1H), 4.66 (dd, *J* = 5.3, 2.9 Hz, 1H), 4.62 – 4.43 (m, 4H), 3.87 (dd, *J* = 9.2, 3.7 Hz, 1H), 3.73 – 3.61 (m, 3H), 2.58 – 2.38 (m, 1H), 2.38 – 2.23 (m, 1H). ¹³C NMR (101 MHz, D₂O) δ 171.7, 167.1, 155.6, 153.1, 149.3, 139.3, 133.6, 130.1, 130.0, 128.5, 128.4, 120.5, 114.5, 90.9, 85.5, 83.9, 83.5, 81.8, 79.6, 58.8, 55.8, 52.9, 52.2, 50.7, 29.7, 28.5, 28.1, 27.3, 25.5. HRMS: calculated for C₂₂H₂₈N₈O₆ [M+H]⁺ 501.2205; found 501.2223.

Enzymatic activity assay

The enzyme was cloned, expressed and purified as previously described.¹⁵ Purity of the enzyme was confirmed using SDS-PAGE with Coomassie blue staining (see supplemental figure S1) and NNMT identity was confirmed using SDS-PAGE and Western blotting. Catalytic activity of recombinant protein was evaluated as previously reported¹⁵ with 1 unit of enzyme activity representing the formation of 1 nmol of MNA per hour of incubation at 37 °C. The homogeneous recombinant NNMT specific activity was 10260 units/mg of protein at a protein concentration of 1.1 mg/mL. NNMT was used at a final concentration of 150 nM in a mixture containing 50 mM Tris Buffer (pH 8.6) and 1 mM DTT. The compounds were incubated with the enzyme at room temperature for 10 minutes before initiating the reaction with a mixture of NA and AdoMet at their K_M values of 200 μM and 8.5 μM respectively. The concentration of inhibitor ranged from 1 μM to 1 mM (DMSO was kept below 5% final concentration). The formation of MNA was measured after 30 minutes at 37°C. The reaction was quenched by addition of 15 μL sample to 70 μL acetonitrile containing 5 μM deuterio-methylated nicotinamide as internal standard. The enzymatic activity assays were performed using UHP-HILIC-MS as previously described with minor modifications.¹⁶ UHP-HILIC-MS analysis was performed using a 1290 Infinity UHPLC system (Agilent Technologies, Waldbronn, BW, Germany) consisting of a binary pump, an autosampler and a temperature controlled column department at 65°C with a Waters (Milford, MA, USA) acquity UPLC BEH amide 1.7 μm 3.0x100 mm HILIC column coupled to a Q-TOF II mass spectrometer with an electrospray ionization source and liquid chromatography sprayer from Bruker Daltonics (Bremen, HB, Germany), operated in positive mode. The analytes were isocratically eluted using 20% water containing 300 μM formic acid and 550 μM NH₄OH (pH 9.2)

and 80% acetonitrile with a flow-rate of 0.6 mL/min and a run-time of 2.6 minutes. The injection volume was 10 μ L and the spectra sample rate was 0.8 Hz. For the analysis of the inhibitory activity of MNA, 4MeNA was used as a substrate at its K_M concentration of 400 μ M.¹⁶ The other parameters in the assay were kept the same. The enzymatic activity was determined by measurement of the formation of MNA. The results were normalized with the “no inhibitor” control set at 100% activity. The “no inhibitor” controls were independently performed for each IC_{50} determination. The measured activities were plotted as a function of inhibitor concentration. The “no inhibitor” control was plotted at 0.1 μ M, 10-fold lower than the lowest concentration measured. Plotting the “no inhibitor” control at 0.01 μ M or 0.001 μ M had no effect on the curve fit or the IC_{50} data generated.

The data was fitted using non-linear regression analysis of the Sigmoidal dose-response curve generated using normalized data and a variable slope following equation 1;

$$Y = \frac{100}{(1 + 10^{((\text{Log}IC_{50} - X) * \text{HillSlope}))}} \quad \text{Equation [1]}$$

where Y = percent activity, X = the logarithmic concentration of the compound and Hill Slope = slope factor or Hill coefficient. The IC_{50} value was determined by the concentration resulting in a half-maximal percent activity. Values are reported along with standard errors of the mean (S.E.M., calculated using the symmetrical CI function in Graphpad Prism 6) indicating the precision of the mean values obtained.

Modelling studies

Compound **45** was built in Yasara version 16.9.23²⁸, based on the AdoHcy and NA structure in complex with NNMT (PDB-ID: 3ROD).¹⁵ The model was energy minimized with the steepest descent method using the Amber14 forcefield²⁹, before analysing the hydrogen bond interactions with Yasara (Fig. 4).

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